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<p>(21) International Application Number: PCT/GB84/00220 (22) International Filing Date: 22 June 1984 (22.06.84) (31) Priority Application Number: 8317124 (32) Priority Date: 23 June 1983 (23.06.83) (33) Priority Country: GB (71)(72) Applicants and Inventors: EKINS, Roger, Philip [GB/GB]; JACKSON, Thomas, Michael [US/GB]; Department of Molecular Endocrinology, The Middlesex Hospital Medical School, Mortimer Street, London W1N 8AA (GB). (74) Agent: HALE, Stephen, Geoffrey; J.Y. & G.W. Johnson, Furnival House, 14-18 High Holborn, London WC1V 6DE (GB). (81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.</p>		<p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: FREE LIGAND ASSAY</p> <p>(57) Abstract</p> <p>A method of measuring the concentration of a free ligand in a biological fluid containing the free ligand and ligand bound to endogenous binding agent, comprises; a) mixing a sample of the fluid with an analogue of the ligand, a specific binder with which the free ligand and the ligand analogue bind, and an exogenous binding agent which binds the ligand analogue but not the ligand, either the ligand analogue or the specific binder being labelled; b) incubating the resulting mixture so that the ligand and ligand analogue compete for the specific binder; c) determining either the amount of the labelled analogue bound to the specific binder or the exogenous binding agent or the amount of labelled specific binder bound, or not bound, to the ligand analogue; and d) correlating the determined amount to the amount of free ligand present in the sample. The method is especially useful where the ligand analogue binds with endogenous binding agent and the extent of binding varies from sample to sample of the biological fluid. It may be used to measure concentration of free thyroid hormones and other hormones in body fluids, employing antibodies specific to the ligand analogue as the exogenous binding agents.</p>		

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1.
Free Ligand Assay

Technical Field

The present invention relates to a method for measuring the concentration of free ligand in a biological fluid containing free ligand and ligand bound to endogenous binding agent.

Background Art

5 Immunoassay techniques have been developed in recent years to measure concentrations of free hormones and other ligands in sera and other biological fluids which contain free ligand in equilibrium with ligand bound to endogenous binding agents such as binding proteins. They are based on the principle that if a specific binder for the ligand, usually an antibody, is
10 brought into contact with the sample to be tested the extent of occupancy of the binding sites on the specific binder by the ligand is a measure of the concentration of free ligand, provided that the amount of specific binder is sufficiently low that the equilibrium between free and endogenously bound ligand is not significantly affected. By measuring the extent of occupancy
15 for the unknown sample and calibrating such a measurement using standard samples containing known free ligand concentrations it is possible to determine the free ligand concentration in the unknown sample.

Initially, the extent of occupancy of binding sites was measured by removing the specific binder containing bound ligand from the sample and
20 determining the proportion of unoccupied sites by back-titration using an appropriately labelled material (e.g. radioactively labelled material) which binds at the unoccupied sites. The process was thus effectively a two-step process.

Subsequently it has been proposed to carry out the 'back-titration' without
25 removing the specific binder from the sample, thus converting the two-step



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process into a one-step process. This can be done either by using as the labelled material a labelled analogue of the ligand or by using as the labelled material a specific binding agent.

Thus, it has been proposed in published European Patent Application
5 No. 0,026,103 to measure the concentration of free ligand in such a biological fluid by a radioimmunoassay technique comprising a) admixing a sample of the fluid with a labelled derivative of the ligand and with a specific binder for the ligand, b) effecting reaction between the free ligand, the labelled derivative and the specific binder, c) if necessary,
10 separating that portion of the ligand and labelled derivative that has become bound to the specific binder from that portion not so bound, d) measuring the amount of the labelled derivative that is, or is not, bound to the specific binder, and e) using that measurement to determine the concentration of free ligand in the biological fluid. According to the
15 process disclosed there, the labelled derivative of the ligand is chosen to bind strongly to the added specific binder but to bind not at all, or much more weakly than does the ligand, to the endogenous binding agent.

In an alternative procedure a method of determining the free ligand concentration involves an immunoradiometric assay comprising admixing a
20 sample of the fluid with a labelled specific binder and an unlabelled analogue of the ligand, incubating the resulting mixture to permit the free ligand and the unlabelled analogue to compete for the labelled specific binder, determining the amount of labelled specific binder bound either to the ligand or to the unlabelled ligand analogue, and correlating the amount
25 of bound labelled specific binder to the amount of free ligand present in the sample.



However when practical assay kits embodying the principles of EPA0,026,103 have been employed to assay free thyroid hormone in samples taken from patients suffering, for example, from certain non-thyroidal illnesses or having serum protein abnormalities unrelated to free thyroid hormone concentration, the assay results appear to show an anomalous free thyroid hormone concentration, contrary to the correct position. It has also been found that the concentration of antibody (acting as specific binder) in those kits can be up to 100 times greater than would have been expected on the simplified theoretical explanation of this technique hitherto proposed.

Further investigation into the operation of those kits has revealed that, far from the ligand analogue being totally unbound to endogenous binding agents or being bound to only a small extent, it is bound to a very substantial extent, at least 90% and probably as much as 99%, not only to the albumin present in the sample but also to the other binding proteins TBG and TBPA.

It is therefore an object of the present invention to devise an alternative and improved technique for assaying free ligand concentrations which is not subject to the disadvantages inherent in the previous technique.

Disclosure of Invention

It has now been found that, for the immunoassay technique using labelled ligand analogue, when any fraction of the ligand analogue becomes bound to other binding agents in the sample as well as to the specific binder the fraction, b , of the ligand analogue bound to the specific binder is represented by the following equation (provided that the concentration of specific binder is sufficiently low that the equilibrium between free and bound ligand in the sample is not significantly disturbed):



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$$b^2 - b \left(\frac{K_H [fH] (1 + \sum K_P [P])}{K_{An} [An]} + \frac{1 + \sum K_P [P]}{K_{An} [An]} + \frac{[Ab]}{[An]} + 1 \right) + \frac{[Ab]}{[An]} = 0$$

where,

K_H is the equilibrium constant for the ligand/specific binder reaction,

5 K_{An} is the equilibrium constant for the analogue/specific binder reaction,

$[fH]$ is the free ligand concentration,

$[An]$ is the analogue concentration,

$[Ab]$ is the specific binder concentration,

$$\sum K_P [P] \text{ is } K_{P_1} [P_1] + K_{P_2} [P_2] \dots + K_{P_n} [P_n],$$

10 $[P_1], [P_2] \dots [P_n]$ are the concentrations of the various (endogenous or added) binding agents other than Ab in the sample, and

$K_{P_1}, K_{P_2} \dots K_{P_n}$ are the corresponding equilibrium constants for the reactions between the ligand analogue and the various (endogenous or
15 added) binding agents.

A similar equation, in which the term $1 + \sum K_P [P]$ - hereafter referred to as S - also appears, can be formulated for the immunometric assay technique in which labelled binding agent is used.

On the basis of this theoretical equation it is possible to design an
20 improved assay technique.

Firstly, in situations where the ligand analogue binds with endogenous binding agent and the extent of binding varies from sample to sample of



the biological fluid because of variations in the concentration of endogenous binding agent and/or the equilibrium constant for the analogue/endogenous binding agent reaction, it is possible to reduce the significance of those variations by adding a further binding agent (X) having a concentration $[P_x]$ and an equilibrium constant K_{P_x} for reaction with the analogue such that $K_{P_x} [P_x]$ contributes significantly to the term S and the contribution to that term from the products $K_{p_1} [p_1] \dots K_{p_n} [p_n]$ for the endogenous binding agent(s) is proportionately reduced.

Secondly, even in situations where the ligand analogue does not bind at all or to any significant extent with endogenous binding agent present in the sample or where the concentrations and equilibrium constants for endogenous binding agent do not vary significantly from sample to sample, the addition of a further binding agent for the ligand analogue enables the term S to be increased. The constraints imposed by the equation then allow $[Ab]$ to be increased without sacrificing the accuracy or responsiveness of the technique, which means that the assay can be completed more quickly or that it is possible to use a specific binder of lower specific activity.

According to the invention therefore there is provided an improved method of measuring the concentration of a free ligand in a biological fluid containing the free ligand and ligand bound to endogenous binding agent, comprising

- a) mixing a sample of the fluid with an analogue of the ligand, a specific binder with which the free ligand and the ligand analogue bind, and an exogenous binding agent which binds the ligand analogue but not the ligand, either the ligand analogue or the specific binder being labelled,



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- b) incubating the resulting mixture so that the ligand and ligand analogue compete for the specific binder,
- c) determining either the amount of the labelled analogue bound to the specific binder or the exogenous binding agent or the amount of labelled specific binder bound, or not bound, to the ligand analogue, and
- d) correlating the determined amount to the amount of free ligand present in the sample.

The exogenous binding agent functions in the method of the present invention as a buffering system for the ligand analogue, having the effect of reducing or eliminating irrelevant fluctuations in the composition of the fluids being tested. Essentially the ligand analogue is the subject of competition between two binding agents, namely the specific binder - hereafter referred to as A - which is also a binder for the ligand, and the exogenous binding agent - hereinafter referred to as X - which is not a binder for the ligand.

Where the contribution to S from the endogenous binding agents is zero or is substantially constant for all samples the exogenous binding agent X and its concentration are advantageously chosen so that $K_{p_x} [P_x]$ and S are both at least 10, S preferably being 50-500. When the contribution to S from the endogenous binding agents is liable to vary significantly from sample to sample the exogenous binding agent X and its concentration are advantageously chosen so that $K_{p_x} [P_x]$ is comparable with or larger than the expected variation in S and preferably constitutes at least half of S, for example two thirds to nine tenths of S.



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It will be appreciated however that the ranges of optimum utility for the product $K_{p_x} [p_x]$ as a fraction of S will vary from case to case depending on the clinical acceptability of inaccuracies in the measurement of free ligand and the extent to which the contribution to S from the endogenous binding agents alone is likely to vary.

A practical upper limit on the amount of exogenous binding agent X may often be imposed by the fact that increases in the amount of the exogenous binding agent X will in general be accompanied by increases in the amount of the specific binder A and that too great an increase in the amount of the specific binder A will lead to a significant disturbance of the equilibrium between free and bound ligand in the biological fluid.

Expressed in terms of competing equilibrium reactions, the method of the present invention can be depicted as shown in Fig.1 of the accompanying drawings. An equilibrium is set up between free ligand, endogenously bound ligand, ligand bound to specific binder A, free ligand analogue, ligand analogue bound to specific binder, ligand analogue bound to exogenous binding agent X and, in the usual case, endogenously bound ligand analogue. Thus the invention differs from the system described in EPA0,026,103 by the provision of a ligand analogue which can be extensively bound to endogenous binding agents and is buffered by the presence of the additional exogenous binding agent X so that the effects of fluctuations in the equilibrium reactions with endogenous binding agents can be proportionately reduced.

The choice of the exogenous binding agent X is dependent upon the nature of the ligand and the ligand analogue because it is essential that it should



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bind with the ligand analogue and not with the ligand. It is also an essential requirement for this binding agent, as for the specific binder A, that it must not through its inherent nature or its concentration disturb the equilibrium between the free ligand and the endogenously bound ligand, nor must it in turn be influenced by the endogenous binding agent or by drugs or any other ingredients likely to be present in the fluids being tested. The exogenous binding agent X may for example be a physical encapsulation of the analogue. Preferably, however, it will be a reagent, especially an antibody, which is tailored according to the chemical differentiation between the ligand and analogue so as to bind the latter but not the former. Advantageously, the antibody acting as binding agent X does not have a very high affinity for the analogue (provided that its affinity for the ligand is lower by at least about 2 orders of magnitude). Such antibodies may then be used at fairly high concentrations to provide the required value for the product $K_{p_x} [p_x]$. Those familiar with immunoassay techniques will be able to design an appropriate exogenous binding agent X without difficulty.

The method of the present invention is applicable not only to immunoassay techniques (eg. radioimmunoassay) in which the ligand analogue is labelled (eg. radioactively) but also to immunometric assay techniques (e.g. immunoradiometric assays) in which the specific binder is labelled (e.g. radioactively).

The method may be used to measure concentrations of free hormones in biological fluids, especially free thyroid hormones T_3 and T_4 but also other hormones such as cortisol, progesterone, oestradiol and testosterone. The



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specific binders A used may be those known to be useful for this purpose in previous immunoassay techniques or may be formulated according to known principles. The ligand analogues described in EPA 0,026,103 and 0,073,865 may be used in the method of the present invention, as may other ligand analogues and it will be appreciated that it is no longer necessary to attempt to design a ligand analogue which will not be bound to endogenous binding agents but merely one which can be bound to a binding agent X which does not bind the ligand itself. The ligand analogues can be labelled in any appropriate manner, for example as described in EPA 0,026,103 when immunoassay techniques are to be used. Alternatively, the analogues may be used in an unlabelled state together with a labelled specific binder as described in greater detail in International patent application WO 83/03306. The other operational conditions appropriate for the method of the present invention may be the same as those known or conventional in previous immunoassay techniques.

The invention and the improvement achievable by its use, are illustrated by the following example.

Example

An analogue of thyroxine (T_4) suitable for the immunoassay of free T_4 (fT_4) as described in EPA 0,026,103 was prepared by chemical modification of the amino acid structure of T_4 . An antibody (X) against this analogue was produced by well known immunological techniques and shown to have a relative affinity for analogue as compared to its affinity for T_4 of 10^3 .



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The analogue was radiolabelled with ^{125}I by the well known "exchange" method and shown to have much lower affinity constants than T_4 for the normal T_4 binding proteins thus satisfying the requirements of EPA 0,026,103.

- 5 A specific antibody against T_4 (A), with an equal affinity for the modified T_4 analogue was coupled to solid particles.

A mixture was prepared of 0.5 ml of a suspension of the solid-phase antibody reagent (2 nm) and 0.5 ml of the ^{125}I T_4 analogue (2 nM), both diluted in 4% BSA, PBS pH 7.4 and a 100 μl aliquot of normal human serum
10 containing various concentrations of fT_4 (prepared by well known techniques). The extent of binding of the ^{125}I analogue to the specific binding reagent was correlated with fT_4 concentration as shown in Fig. 2(a).

A similar mixture was prepared containing identical concentrations of specific antibody and analogue but with 100 μl aliquots of samples con-
15 taining 3 mM oleic acid and varying concentrations of fT_4 . (Oleic acid is one of a class of compounds known as non-esterified-fatty acids which are known to be increased in serum samples following the administration of some drugs and during non-thyroidal illness.)

The extent of binding was found to correlate with fT_4 as shown in Fig. 2(b).
20 Thus a sample containing, e.g., 20 pM fT_4 and 3 mM oleic acid would, because of the increase in the extent of analogue binding, be interpreted as containing 10.6 pM fT_4 , a bias of 47%.



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The $K_{p_n}[P_n]$ in the incubation conditions of this assay is estimated to be 70.

According to the method of this invention, the additional binding agent prepared as previously described, was added to identical mixtures of 5 antibody and antigen at a concentration of 0.2% such that $\sum K_x[P_x] = 150$. Again the extent of binding of the labelled analogue with the specific antibody was correlated with the fT_4 concentration before and after the addition of 3 mM oleic acid as shown in Fig. 2(c and d) respectively.

10 In this example of the invention a sample containing 20 pg/ml fT_4 and 1 mM oleic acid would be interpreted as containing 17 pg/ml fT_4 , a negative bias of only 15%. Further increases in the addition of the binding agent cause additional decreases in the observed bias of the method and the required concentration of binding agent will depend on the bias permissible in the estimation of fT_4 for clinical reasons.



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CLAIMS

1. A method of measuring the concentration of a free ligand in a biological fluid containing the free ligand and ligand bound to endogenous binding agent, comprising

5 a) mixing a sample of the fluid with an analogue of the ligand, a specific binder with which the free ligand and the ligand analogue bind, and an exogenous binding agent which binds the ligand analogue but not the ligand, either the ligand analogue or the specific
10 binder being labelled,

b) incubating the resulting mixture so that the ligand and ligand analogue compete for the specific binder,

15 c) determining either the amount of the labelled analogue bound to the specific binder or the exogenous binding agent or the amount of labelled specific binder bound, or not bound, to the ligand analogue, and

d) correlating the determined amount to the amount of free ligand present in the sample.

20 2. A method as claimed in claim 1 wherein the contribution from the endogenous binding agents to S (defined as one plus the sum of the products of the concentrations of each of the binding agents in the sample other than the specific binder and the respective equilibrium con-
25 stants for the reactions between those binding agents and the ligand analogue) is zero or substantially constant for all samples and the exogenous binding agent and its concentration are selected so that its contribution to S is at least 10, S being not more than 500.

30 3. A method as claimed in claim 1 wherein the contribution from the endogenous binding agents to S (defined



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as in claim 2) varies significantly from sample to sample and the exogenous binding agent and its concentration are chosen so that its contribution to S is at least half the value of S in any sample and is comparable with
5 or greater than the variation in S from sample to sample.

4. A method as claimed in claim 1 wherein the exogenous binding agent is a chemical reagent which binds the ligand analogue but does not bind the ligand to a significant extent.

10 5. A method as claimed in claim 4 wherein the exogenous binding agent is an antibody having an affinity for the ligand analogue at least two orders of magnitude higher than its affinity for the ligand.

15 6. A method as claimed in claim 1 wherein the ligand analogue is labelled and an immunoassay technique is used.

7. A method as claimed in claim 1 wherein the specific binder is labelled and an immunometric assay technique is used.

20 8. A method as claimed in claim 1 wherein the label is a radioactive label.

9. A method as claimed in claim 1 wherein the free ligand is free hormone.

25 10. A method as claimed in claim 9 wherein the free ligand is free thyroid hormone.



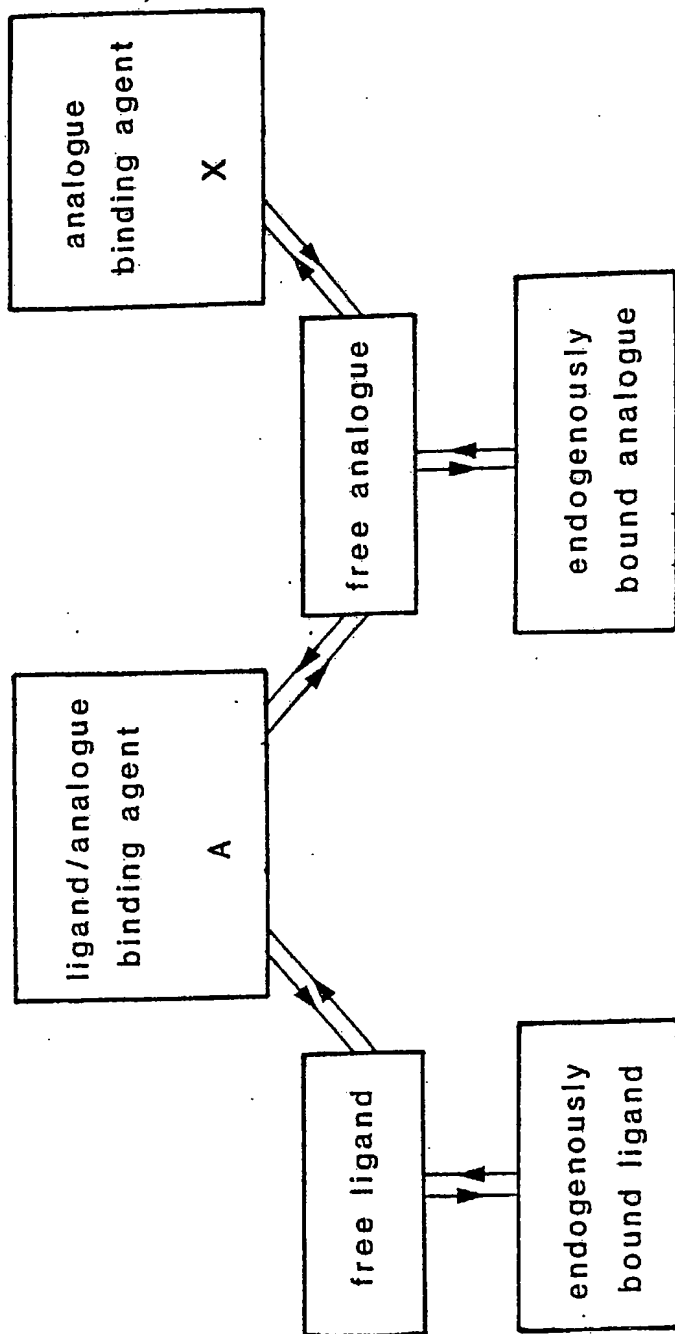
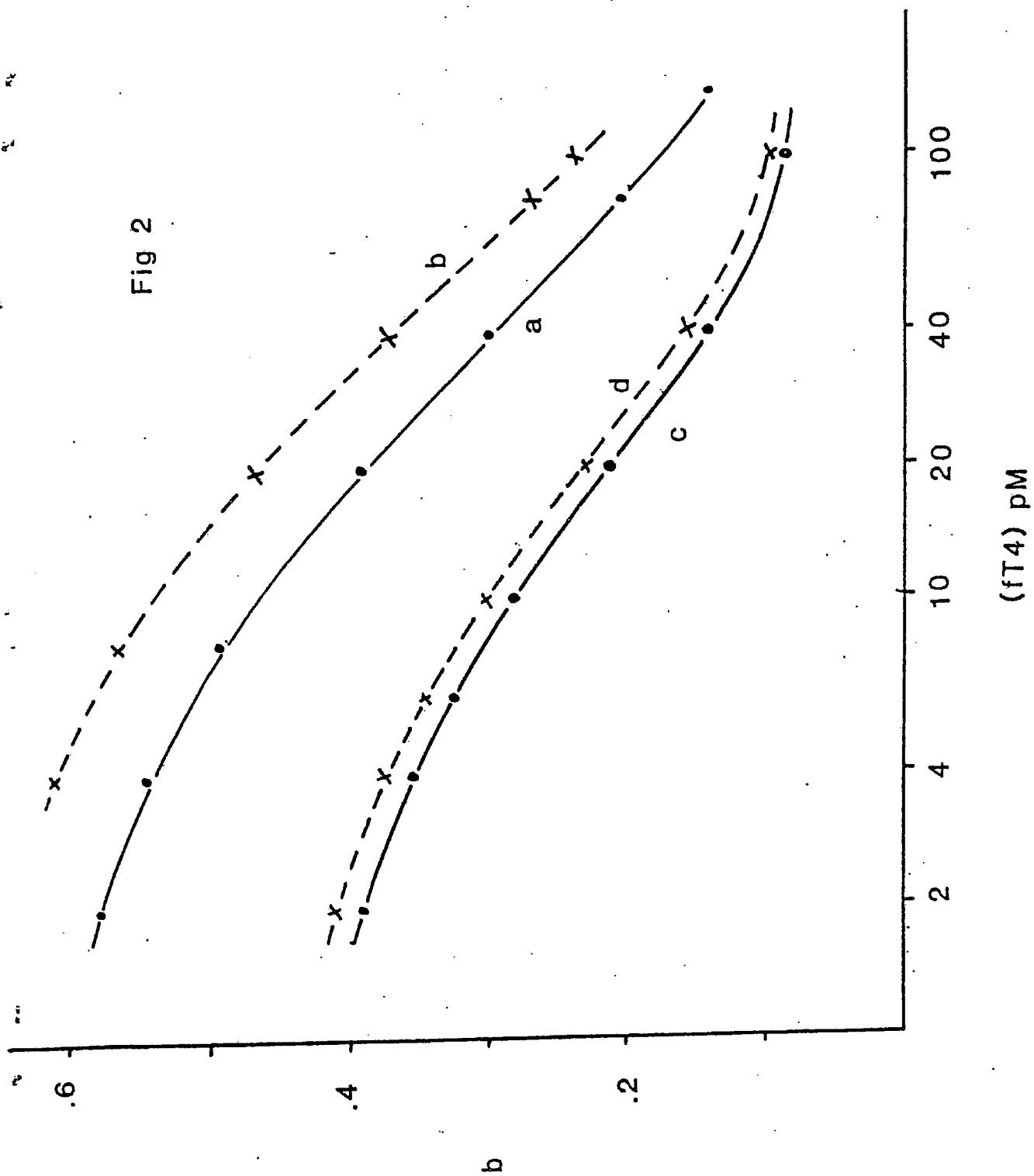


Fig 1

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Fig 2



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 84/00220

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ³ : G 01 N 33/53; G 01 N 33/78		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
IPC ³	G 01 N	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁴		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁵	Citation of Document, ⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
P, X	WO, A, 83/03306 (R.P. EKINS) 29 September 1983 see the entire document (cited in the application) --	1,5-10
P, A	WO, A, 84/01031 (R.P. EKINS) 15 March 1984 see the entire document --	1,5-9
A	EP, A, 0073865 (AMERSHAM INTERNATIONAL PLC.) 16 March 1983 see pages 2a-4 and examples 4,5; claims 9,10 (cited in the application) --	1,5-10
A	EP, A, 0026103 (THE RADIOCHEMICAL CENTRE LTD.) 1 April 1981 (cited in the application) --	
A	GB, A, 2085160 (CORNING GLASS WORKS) 21 April 1982	
<p>* Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, each combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ¹	Date of Mailing of this International Search Report ²	
11th October 1984	06 NOV. 1984	
International Searching Authority ¹	Signature of Authorized Officer ²⁰	
EUROPEAN PATENT OFFICE	G.L.M. Knudsenberg	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁴
A	EP, A, 0015687 (R.P. EKINS) 17 September 1980 -----	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 84/00220 (SA 7438)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 26/10/84

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8303306	29/09/83	EP-A- 0103605	28/03/84
WO-A- 8401031	15/03/84	AU-A- 1944283	29/03/84
EP-A- 0073865	16/03/83	None	
EP-A- 0026103	01/04/81	JP-A- 56051665	09/05/81
		US-A- 4366143	28/12/82
		CA-A- 1144477	12/04/83
		AU-B- 528427	28/04/83
GB-A- 2085160	21/04/82	FR-A- 2490826	26/03/82
		JP-A- 57086052	28/05/82
		DE-A- 3136579	19/08/82
		US-A- 4410633	18/10/83
EP-A- 0015687	17/09/80	JP-A- 55146043	14/11/80
		US-A- 4381291	26/04/83

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